

## Expression of catalase and glutathione peroxidase in renal insufficiency

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### Abstract

Chronic renal failure (CRF) is associated with oxidative stress, the precise mechanism of which is yet to be elucidated. The present study was undertaken to investigate in renal insufficiency the expression of catalase and glutathione peroxidase, which play a critical role in antioxidant defense system by catalyzing detoxification of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and organic hydroperoxides. Rats were randomly assigned to the CRF (5/6 nephrectomized) and sham-operated control groups and observed for 6 weeks. Renal and thoracic aortic catalase and glutathione peroxidase protein abundance was measured by Western blotting. The enzyme activities in the renal and aortic extracts, hepatic glutathione levels, blood pressure and urinary nitric oxide metabolites (NO<sub>x</sub>) excretion were also measured. Blood pressure and urinary nitric oxide metabolite (NO<sub>x</sub>) excretion were also measured. The CRF group showed a significant down-regulation of both immunodetectable catalase and glutathione peroxidase proteins in the remnant kidney. Catalase activity was also significantly decreased in the remnant kidney whereas glutathione peroxidase activity was not significantly affected. Furthermore, the protein abundance of catalase was unchanged whereas the enzyme activity was significantly decreased in the thoracic aorta of CRF animals compared to the sham-operated controls. By contrast, both the protein abundance and the enzyme activity of glutathione peroxidase were not significantly affected in the aorta of CRF animals compared to the sham-operated controls. This was coupled with marked arterial hypertension, significant reduction of hepatic glutathione levels and urinary NO<sub>x</sub> excretion pointing to increased inactivation and sequestration of NO by superoxide. These events point to the role of impaired antioxidant defense system in the pathogenesis of oxidative stress in CRF.

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### 1. Introduction

Chronic renal failure (CRF) is associated with oxidative stress that contributes to the development of numerous short- and long-term complications including hypertension, anemia, arteriosclerotic cardiovascular disease, neurological disorders, hemostatic abnormalities and impaired immunity. The presence of oxidative stress in CRF is evidenced by

overabundance of byproducts of interaction of reactive oxygen species (ROS) with various functional or structural molecules in the plasma and tissues of CRF humans and animals. For instance, several studies have shown elevated plasma concentration of lipid peroxidation product, malondialdehyde, in humans and animals with CRF [1–4]. Similarly, plasma concentrations of glycoxidation (AGE) and lipoxidation end products are elevated in CRF denoting increased ROS-mediated generation of reactive carbonyl (RCO) compounds and their subsequent reaction with plasma proteins [5,6]. In addition, we have recently found enhanced inactivation and sequestration of nitric oxide (NO)

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by ROS leading to functional NO deficiency, hypertension and accumulation of nitrated proteins in various tissues of CRF rats [7,8].

While the presence of oxidative stress as a feature of CRF is well established, its underlying mechanisms remain incompletely understood. In a recent study of CRF rats [9], we found marked up-regulation of nicotinamide adenine dinucleotide phosphate [NAD(P)H] oxidase which is the major source of superoxide production. This was coupled with significant down-regulation of cytoplasmic (CuZn SOD) and mitochondrial (Mn SOD) superoxide dismutase in the liver and kidney of CRF animals. Increased NAD(P)H oxidase and reduced cellular SOD can work in concert to raise superoxide abundance. This was substantiated by overabundance of nitrotyrosine, which is the footprint of superoxide–NO–tyrosine interaction in various tissues of the CRF rats. The functional significance of excess superoxide abundance was verified by favorable response to the administration of the SOD-mimetic agent, tempol, which mitigated NO inactivation by superoxide, increased NO availability and ameliorated hypertension in CRF animals [9].

SOD catalyzes dismutation of superoxide to hydrogen peroxide. Hydrogen peroxide, in turn, is converted to water and molecular oxygen by catalase or glutathione peroxidase (GPX) which uses glutathione as a substrate.

The present study was undertaken to examine the effect of CRF on renal and thoracic aortic catalase and glutathione peroxidase, as well as hepatic glutathione levels. The results showed marked down-regulation of renal catalase protein abundance as well as the enzyme activity in CRF rats. In thoracic aortic extracts, catalase activity was significantly decreased in the animals of CRF group compared to the controls whereas GPX activity was unchanged. In addition, hepatic glutathione levels and urinary NO<sub>x</sub> excretion were depressed in CRF rats.

## 2. Methods

### 2.1. Study groups

Male Sprague–Dawley rats weighing 230–250 g (Harlan Sprague Dawley Inc., Indianapolis, IN) were used in this study. The animals were housed in a climate-controlled vivarium with 12-h day and night cycles and were provided rat chow and water ad libitum. The animals were randomly assigned to the CRF and sham-operated control groups (8 animals/group). The CRF group underwent 5/6 nephrectomy by surgical resection of the upper and lower thirds of the left kidney followed by right nephrectomy 4 days later. The control group underwent sham operation. The procedures were carried out under general anesthesia (pentobarbital 50 mg/kg, IP) using strict hemostasis and aseptic techniques. The nephrectomy procedures were accomplished via dorsal incisions as described in our earlier

publications [10,11]. The animals were monitored for 6 weeks after nephrectomy and sham operation.

At the conclusion of the 6-week observation period, the animals were placed in individual metabolic cages for a timed-urine collection. The animals were anesthetized (pentobarbital 50 mg/kg, IP) and exsanguinated by cardiac puncture. Remnant kidney and thoracic aorta were immediately harvested, cleaned and snap frozen in liquid nitrogen. Plasma and frozen tissues were then stored at –70 °C until processed. The experimental protocol employed in the study was approved by the Institutional Animal Care and Use Committee of the University of California, Irvine. Blood pressure, body weight, creatinine clearance, hematocrit and urinary excretion of nitrate and nitrite (NO<sub>x</sub>) were determined in the study animals.

### 2.2. Preparation of tissue extracts

Homogenates (25% w/v) of kidney and thoracic aorta were prepared in 10 mM *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid (HEPES) buffer, pH 7.4, containing 320 mM sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 10 µg/ml leupeptin, 2 µg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride (PMSF) at 4 °C using a Polytron homogenizer. Homogenates were centrifuged at 9000×*g* for 10 min at 4 °C to remove nuclear fragments and tissue debris without precipitating membrane fragments. The supernatants thus obtained were used for measuring the enzyme activities, glutathione content as well as for Western blot analyses. A portion of the supernatant was used for the determination of total protein concentration by using a Bio-Rad kit (Hercules, CA).

### 2.3. Western blot analyses

Total cellular protein (20 µg each) was electrophoresed in 4–20% Tris–glycine SDS polyacrylamide gels (Novex, San Diego, CA) as detailed in our earlier publication [12]. Proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore Corp., Bedford, MA), blocked in 5% dry milk in Tris–buffered saline (T-TBS) containing 0.1% Tween 20 (0.02 M Tris/0.15 M NaCl, pH 7.5) at room temperature for 3 h, washed 3× with T-TBS and incubated with the primary antibodies (catalase, 1:2000; GPX, 1:250) for 3 h at room temperature. Catalase and GPX antibodies were purchased from Calbiochem Inc. (San Diego, CA) and Cortex Biochem Inc. (San Leandro, CA), respectively. After washing 5× with T-TBS, the blots were incubated with secondary antibodies (1:2000; anti-rabbit for catalase and anti-sheep for GPX) conjugated with horseradish peroxidase at room temperature for 2 h. After washing 5× with T-TBS, the membrane was developed using enhanced chemiluminescent (ECL) reagent (Perkin Elmer Life Sciences, Boston, MA) and subjected to autoluminography. The autoluminographs were scanned with a laser densitometer (Model PD 1211, Molecular

Dynamics, Sunnyvale, CA) to determine the relative optical densities of the bands.

#### 2.4. Determination of catalase and GPX activities

Catalase activity was measured by the method described by Claiborne [13]. The enzyme activity is defined as micromoles of hydrogen peroxide consumed per minute.

GPX activity was measured by using Bioxytech GPx-340™ kit obtained from Oxis International, Inc. (Portland, OR). The enzyme activity is defined as nanomoles of NADPH consumed per minute.

#### 2.5. Measurement of urinary NO metabolites (NO<sub>x</sub>)

Urinary concentration of NO<sub>x</sub> (total NO<sub>2</sub> and NO<sub>3</sub>) was determined by means of a Sievers model 270B nitric oxide analyzer (Sievers Instruments, Boulder, CO, USA) as described earlier [10].

#### 2.6. Measurement of arterial pressure

Arterial pressure was determined by tail plethysmography (Harvard Apparatus, Natick, MA, USA) as described in our earlier studies [4].

#### 2.7. Measurement of total glutathione

Hepatic levels of total glutathione were determined by using the Bioxytech GSH-400™ kit obtained from Oxis.

#### 2.8. Data analysis

Analysis of variance (ANOVA) and Student's *t*-test were used in statistical evaluation of the data that are presented as mean ± S.E. *P* values <0.05 were considered significant.

### 3. Results

#### 3.1. General data

The CRF group exhibited a significant reduction in creatinine clearance and a marked increase in arterial pressure (Table 1). Mean body weight obtained at the end

Table 1

Body weight, systolic blood pressure (SBP), urinary nitrate and nitrite (NO<sub>x</sub>) excretion, creatinine clearance (Ccr) and hematocrit in the sham-operated control (CTL) and 5/6 nephrectomized (CRF) rats

|                                     | CTL       | CRF        | <i>P</i> values |
|-------------------------------------|-----------|------------|-----------------|
| Body weight (g)                     | 404 ± 5   | 358 ± 8    | <0.01           |
| SBP (mm Hg)                         | 119 ± 3   | 164 ± 4    | <0.01           |
| NO <sub>x</sub> (μmol/g creatinine) | 608 ± 83  | 262 ± 52   | <0.01           |
| Ccr (ml/min)                        | 3.7 ± 0.2 | 1.3 ± 0.03 | <0.01           |
| Hematocrit (%)                      | 43 ± 1.3  | 33 ± 3.1   | <0.01           |

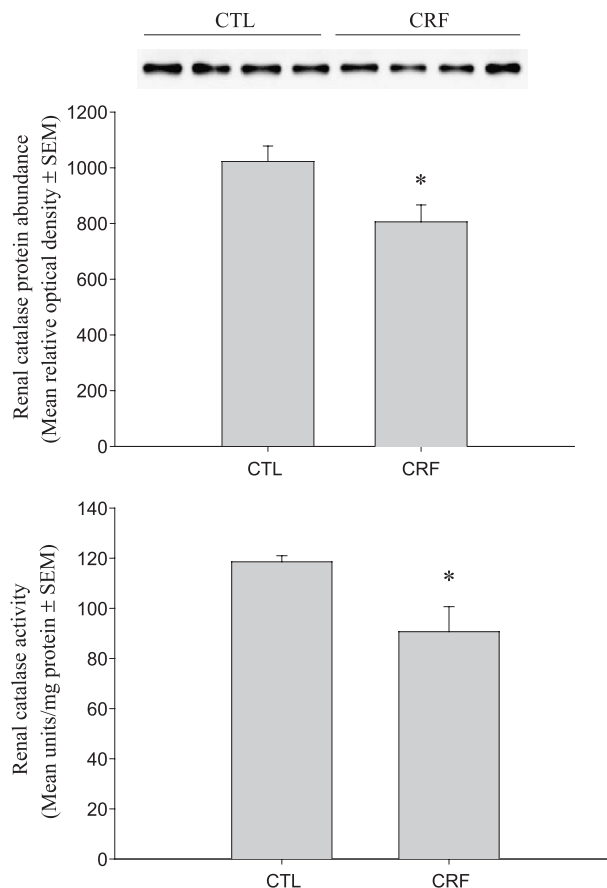


Fig. 1. Representative Western blot and group data depicting catalase protein abundance (upper panel) and the enzyme activity (lower panel) in the kidney of rats with chronic renal failure (CRF) and sham-operated control (CTL) group (*P* < 0.05). *n* = 8 for each group.

of the observation period was significantly lower in the CRF group as compared with the normal control group. Urinary NO<sub>x</sub> excretion was markedly reduced in the CRF animals when compared with the sham-operated controls. Likewise, hematocrit was significantly lower in the CRF group than that found in the sham-operated controls.

#### 3.2. Renal catalase and GPX protein abundance and activities

The CRF group exhibited a significant reduction in renal immunoreactive catalase protein abundance when compared with the control group (Fig. 1, upper panel). Similarly, catalase activity was also significantly decreased in the renal extracts of the animals of CRF group compared to the sham-operated controls (Fig. 1, lower panel).

As with catalase, the immunoreactive GPX protein abundance was significantly decreased in the renal extracts of the animals of CRF group as compared with the control group (Fig. 2, upper panel). However, GPX enzyme activity was not significantly changed in the CRF group compared to the values found in the control group (Fig. 2, lower panel).

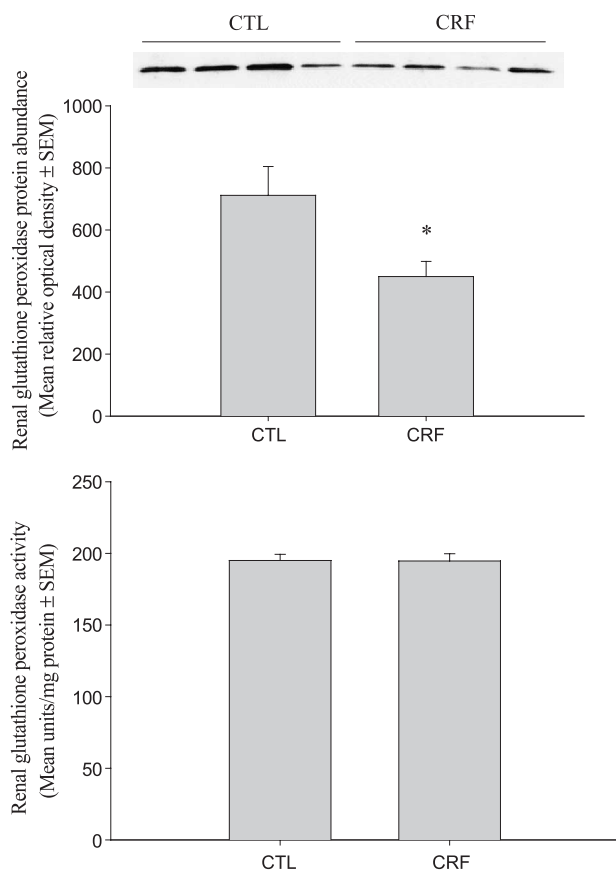


Fig. 2. Representative Western blot and group data depicting glutathione peroxidase protein abundance (upper panel) and the enzyme activity (lower panel) in the kidney of rats with chronic renal failure (CRF) and sham-operated control (CTL) group ( $P < 0.05$ ).  $n = 8$  for each group.

### 3.3. Thoracic aortic catalase and GPX protein abundance and activities

Catalase protein abundance was not significantly changed in the aortic extracts of the animals of CRF group compared to the controls (Fig. 3, upper panel). The enzyme activity, however, was significantly decreased in the aortic extracts of CRF animals as compared to the controls (Fig. 3, lower panel).

GPX protein abundance (Fig. 4, upper panel) as well as the enzymatic activity (Fig. 4, lower panel) were not significantly changed in the aortic extracts of CRF animals compared to the control group.

### 3.4. Hepatic glutathione content

Animals of the CRF group exhibited a significantly lower total hepatic glutathione content as compared to the sham-operated control group (Fig. 5).

## 4. Discussion

While the presence of oxidative stress in CRF is well established, its underlying mechanisms have only recently

begun to unfold. Oxidative stress can occur either as a result of increased ROS generation, depressed antioxidant system or both. The natural antioxidant system consists of a series of antioxidant enzymes and numerous endogenous and dietary antioxidant compounds that react with and inactivate ROS. The primary ROS produced in the aerobic organisms is superoxide that is a highly reactive and cytotoxic agent [14]. Superoxide is converted to  $H_2O_2$  by a group of enzymes known as superoxide dismutases [14].  $H_2O_2$ , in turn, is converted to water and molecular oxygen by either catalase or GPX. In addition, GPX can reduce lipid peroxides and other organic hydroperoxides which are highly cytotoxic products. Accordingly, SOD, catalase and GPX constitute the principal components of the antioxidant defense system and their deficiencies can cause oxidative stress. The present study was conducted to investigate the effect of CRF on catalase and GPX in the kidney and thoracic aorta.

Catalase is a tetrameric peroxidase enzyme which converts  $H_2O_2$  to water and molecular oxygen. Likewise, using  $H^+$  donors, catalase facilitates reduction of organic hydroperoxide ( $ROOH + AH_2 \rightarrow H_2O + ROH + A$ ). Gene expression of catalase is regulated by  $H_2O_2$  [15]. In animals,  $H_2O_2$  is detoxified by catalase and GPX. Catalase protects the cells from  $H_2O_2$  and plays an important role in the

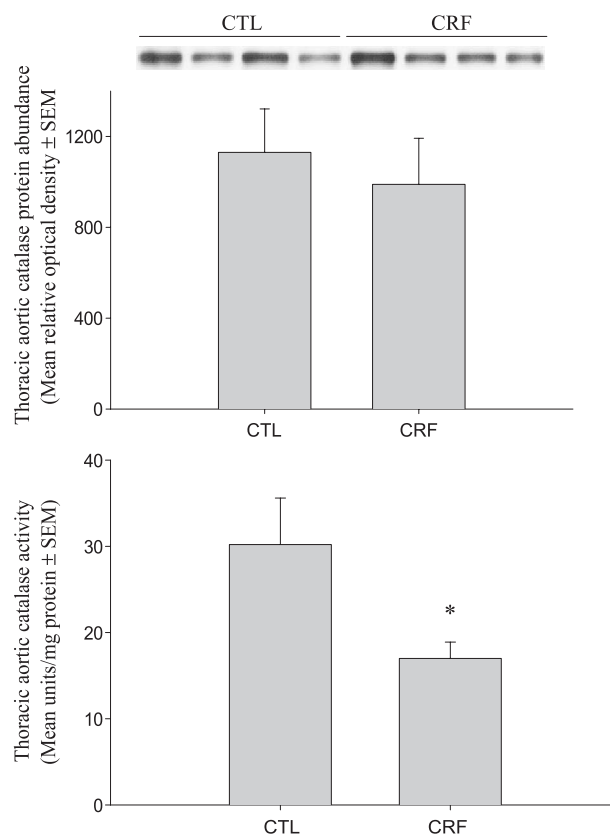


Fig. 3. Representative Western blot and group data depicting catalase protein abundance (upper panel) and the enzyme activity (lower panel) in the thoracic aorta of rats with chronic renal failure (CRF) and sham-operated control (CTL) group ( $P < 0.05$ ).  $n = 8$  for each group.

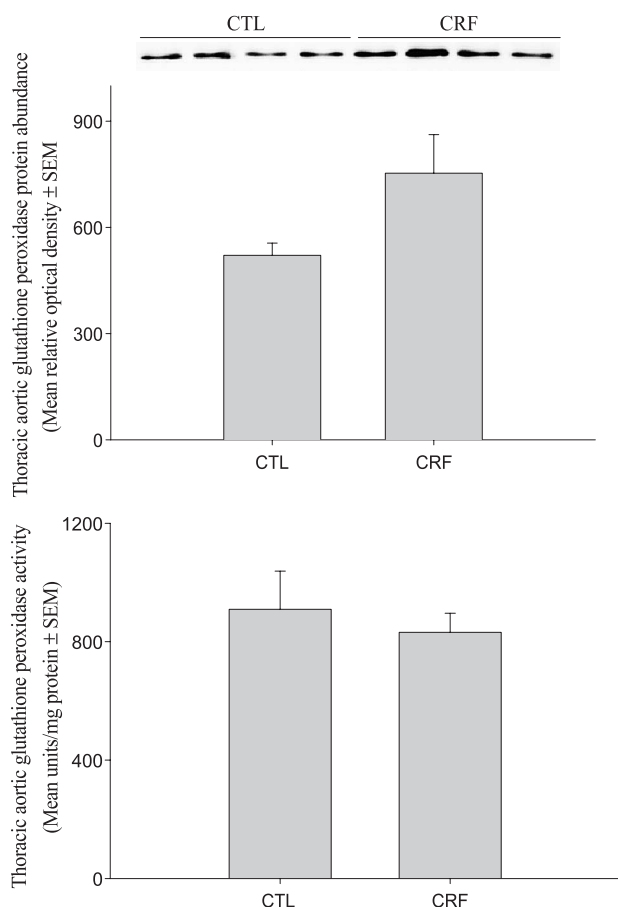


Fig. 4. Representative Western blot and group data depicting glutathione peroxidase protein abundance (upper panel) and the enzyme activity (lower panel) in the thoracic aorta of rats with chronic renal failure (CRF) and sham-operated control (CTL) group ( $P < 0.05$ ).  $n = 8$  for each group.

antioxidant defense system and in adaptation to oxidant stress [15].

The CRF animals employed in the present study exhibited significant reduction of immunodetectable catalase protein abundance as well as the enzyme activity in the kidney. Similarly, in the thoracic aorta, catalase activity was significantly decreased in the CRF animals compared to the normal controls. However, the immunodetectable aortic catalase protein was unchanged in the CRF group. The exact reason for this discrepancy is unclear. However, it could be because of protein modification that may have interfered with its detection by the antibody employed in the present study. Given the critical role of catalase in antioxidant defense system, the observed deficiency of this enzyme in the kidney as well as the aorta must contribute to the CRF-induced oxidative stress. Catalase activity has been reported to be reduced [16,17] or unchanged [18] in the plasma of patients with end-stage renal disease and in the erythrocytes of male Wistar albino rats at 4 weeks after 5/6 nephrectomy [19]. Van den Branden et al. [20] have reported that catalase gene expression in the renal cortex of rat remnant-kidney model of CRF was decreased during development of renal failure and this decrease was accompanied by decreased

catalase activity during the glomerulosclerosis phase of the remnant-kidney model.

Glutathione peroxidase is a selenium-containing tetrameric enzyme which reduces  $H_2O_2$ , lipoperoxides and other organic hydroperoxides to their corresponding hydroxy compounds using glutathione as a hydrogen donor ( $ROOH + 2GSH \rightarrow ROH + GSSG + H_2O$ ). Gene expression of GPX is up-regulated by  $H_2O_2$  and other ROS [15]. Although GPX shares the substrate  $H_2O_2$  with CAT, it alone can react effectively with lipid and other organic hydroperoxides, being the major source of protection against low levels of oxidant stress [15]. Furthermore, GPX and other selenoproteins containing selenocysteine or selenomethionine appear to play a role in the GSH-dependent defense against peroxynitrite-mediated oxidations, by serving as a peroxynitrite reductase [21].

As with catalase, tissue abundance of GPX protein was significantly decreased in the renal extracts of the CRF animals compared with the normal controls. However, the enzyme activity was unchanged in the renal extracts of CRF animals compared to the control animals. In the thoracic aorta, both the GPX protein abundance as well as the enzyme activity were unchanged in the CRF animals as compared to the normal controls. It is of note that Van den Branden et al. [20] have also found that antioxidant enzyme activity and gene expression do not change in the same direction at all times during disease development in the rat remnant-kidney model of CRF.

While GPX activity has been reported to be reduced in plasma of patients with end-stage renal disease [22,23], in red blood cells the enzyme activity in adult patients with end-stage renal disease was comparable to that in the control group [18,20]. However, GPX gene expression remained at a normal level during progression of the disease in the renal cortex of rat remnant-kidney model of CRF during the glomerulosclerosis phase of the remnant-kidney model [20]. By contrast, in the Adriamycin model of CRF, renal cortex GPX activity was decreased to 69% of the

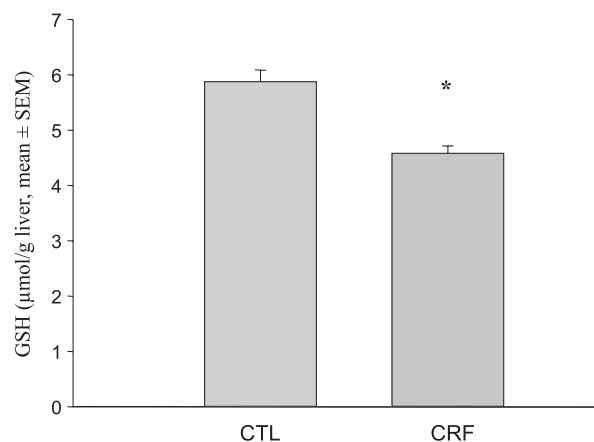


Fig. 5. Hepatic glutathione content in the rats with chronic renal failure (CRF) and sham-operated control (CTL) group ( $P < 0.05$ ).  $n = 8$  for each group.



control levels at 20 weeks after Adriamycin treatment [24]. Similarly, in the erythrocytes of male Wistar albino rats at 4 weeks after 5/6 nephrectomy, GPX activity was found to be reduced [19].

The results obtained in the present study also show a significant reduction of total hepatic glutathione content. In view of the critical role of glutathione in the natural antioxidant defense system, its deficiency may contribute to the associated oxidative stress in the CRF animals.

Several earlier studies have demonstrated the role of oxidative stress in the pathogenesis of hereditary and acquired hypertension [4,7,25–35]. Oxidative stress can raise arterial pressure by several mechanisms of which two are particularly noteworthy: first, increased inactivation and sequestration of NO by ROS leading to functional NO deficiency; second, generation of vasoconstrictive isoprostanes via nonenzymatic oxidation of arachidonic acid [25–29,36]. In fact, we recently demonstrated that induction of oxidative stress by glutathione depletion can cause a severe antioxidant-remediable hypertension marked by avid inactivation and sequestration of NO in genetically normal, otherwise intact animals [31].

Earlier studies from this laboratory have shown that CRF induced by renal mass reduction in rats resulted in oxidative stress as evidenced by a significant increase in nitrotyrosine abundance, a footprint of NO interaction with superoxide, and malondialdehyde concentration in plasma of the animals with renal insufficiency (reviewed in Ref. [25]). This was accompanied by down-regulation of the protein expression of cytoplasmic as well as mitochondrial superoxide dismutases and up-regulation of NAD(P)H oxidase, which together can result in superoxide abundance [9]. These observations were supported by the favorable response to administration of SOD-mimetic drug, tempol, in the CRF animals [9]. The results obtained in the present study show that oxidative stress in CRF may also be at least, in part, due to down-regulation of catalase as well as depletion of glutathione.

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